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(57) **Abstract**

This invention has as its object novel defective adenoviruses for the transfer of genes of interest into a host eukaryotic cell or organism and their expression therein. The invention also relates to novel complementation lines and the process for the preparation of these novel defective adenoviruses, as well as their therapeutic use and a pharmaceutical composition containing them.

* Tr. note: The title and abstract appear in both English and French in the original document. However, the English version provided there is not an accurate translation of the French version. The versions that appear above are our accurate translations from the French.

TITLE PAGE

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DEFECTIVE ADENOVIRUSES AND CORRESPONDING COMPLEMENTATION LINES

The invention has as its object novel defective adenoviral vectors permitting the transfer of genes of interest into a host eukaryotic cell or organism and their expression therein, as well as novel complementation lines complementing *in trans* the essential viral functions that have been deleted from the genome of these recombinant adenoviruses. The invention is of special interest for consideration in gene therapy, notably in man.

The adenoviruses are DNA viruses that have a broad host spectrum. They have been found in numerous animal species and in numerous cell types. There exist several serotypes which differ notably in the sequence of their genomes. The majority of human adenoviruses are not very pathogenic and generally produce only benign symptoms.

The adenovirus penetrates into the permissive host cell via a specific receptor and then it is internalized and passes into endosomes. Their acidification contributes to a conformational change of the virus and to its exit into the cytoplasm. Then, the viral DNA associated with certain viral proteins necessary for the first steps of the replication cycle penetrates into the nucleus of the infected cells, where its transcription is initiated by cellular enzymes. Replication of the DNA takes place in the nucleus of the infected cells and does not require cellular replication. The assembling of new virions also takes place in the nucleus. First, the viral proteins are assembled so as to form empty capsids of icosahedral structure in which the adenoviral DNA is then encapsidated. The viral particles or virions are freed of infected cells and are capable of infecting other permissive cells.

The infectious cycle of the adenovirus occurs in 2 steps:

- the early phase which precedes the initiation of the replication of the adenoviral genome and which permits the production of regulatory

proteins which are involved in the replication and transcription of viral DNA, and

- the late phase which leads to the synthesis of structural proteins.

In general, the adenoviral genome is composed of a linear double-stranded DNA molecule, approximately 36 kb long, which contains sequences coding for more than 30 proteins. At each of its ends there is a short sequence of 100 to 150 nucleotides, depending on the serotype, which is inverted and is designated the ITR (Inverted Terminal Repeat). The ITRs are involved in the replication of the adenoviral genome. The encapsidation region of approximately 300 nucleotides is situated at the 5' end of the genome, just after the 5' ITR.

The early genes are distributed in 4 regions which are dispersed in the adenoviral genome, designated E1 to E4 (E for "Early", meaning early in English). The early regions comprise at least six transcription units which have their own promoters. Expression of the early genes is itself regulated, certain genes being expressed before others. Three regions, E1, E2 and E4 respectively, are essential for viral replication. Thus, if an adenovirus is defective for one of these functions, i.e., if it cannot produce at least one protein coded by one of these regions, the latter will have to be supplied to it *in trans*.

The E1 early region is situated at the 5' end of the adenoviral genome and contains 2 viral transcription units, E1A and E1B respectively. This region codes for proteins which are involved very early in the viral cycle and are essential for the expression of almost all the other genes of the adenovirus. In particular, the E1A transcription unit codes for a trans-activating protein for the transcription of other viral genes, which induces the transcription starting from promoters of the E1B, E2A, E2B and E4 regions.

The products of the E2 region, which also comprises two transcription units, E2A and E2B, are directly involved in viral DNA replication. In particular, this region governs the synthesis of a 72kDa protein, which has a strong affinity for single-strand DNA, and a DNA polymerase.

The E3 region is not essential for virus replication. It codes for at least six proteins that would be responsible for inhibiting the immune response of the host to an infection by the adenovirus. In particular, the gp19kDa glycoprotein would prevent the CTL response which is responsible for cytolysis of infected cells by the cytotoxic T cells of the host.

The E4 region is situated at the 3' end of the adenoviral genome. It codes for numerous polypeptides which are involved in the expression of late genes, the stability of late messengers (mRNAs), the transition from the early phase to the late phase and the inhibition of cellular protein synthesis.

Once the viral DNA replication is initiated, the transcription of late genes begins. The latter occupy the majority of the adenoviral genome and partially cover the transcription units of the early genes. But they are transcribed starting from different promoters and according to an alternative splicing mode, so that the same sequences are used for different purposes. Most of the late genes are transcribed starting from the major late promotor (MLP). This promoter makes possible the synthesis of a long primary transcript which is then matured into about twenty messenger RNAs (mRNAs) from which the capsid proteins of the virion are produced. The gene coding for the IX structural protein composing the capsid is situated at the 5' end of the adenoviral genome and covers the E1B region at its 3' end. The transcription unit of the IX protein uses the same transcription termination signal as the E1B transcription unit.

A certain number of adenoviruses are now well characterized genetically and biochemically. Such is the case for the type 5 human adenovirus (Ad5),

whose sequence is disclosed in the Genebank[1] database under the reference M73260. The various genes have been able to be located precisely on the adenoviral genome which comprises, from 5' toward 3', the 5' ITR of 103 bp followed by the encapsidation region (Hearing et al., 1987, J. Virol., *61*, 2555-2558) of about 300 bp, then the early and late regions whose emplacement is shown diagrammatically in Fig. 1, and finally the 3' ITR.

It follows from the preceding that adenoviruses have interesting characteristics which make them vectors of choice for the transfer of genes of interest. Numerous recombinant adenoviruses are described in the literature (Rosenfeld et al., 1991, Science, *252*, 431-434; Rosenfeld et al., 1992, Cell, *68*, 143-155). In general, they are derived from Ad5 and are defective for the E1 function so as to prevent their dissemination into the environment and the host organism. Moreover, the nonessential E3 region can also be deleted. The exogenous sequences are integrated in place of the E1 or E3 region.

Hence these defective adenoviruses can be propagated only in a cell line complementing *in trans* the E1 function essential for viral replication. At present, the only usable complementation line is the 293 embryonal kidney line (Graham et al., 1977, J. Gen. Virol., *36*, 59-72), which results from the integration into its chromosomes of a fragment of the Ad5 genome comprising notably the 5' end of the viral genome, so that the 293 line complements the adenoviruses that are defective for the E1 function. The 293 cells contain sequences which are also found in the defective recombinant adenovirus, such as the 5' ITR, the encapsidation region and the 3' part of the E1B region comprising sequences coding for the early proteins.

The feasibility of gene transfer using adenoviruses is now established. But the question of their innocuousness remains. In fact, they are capable of transforming certain cell lines in culture, which reflects the potentially

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[1] *Tr. note: Genebank appears throughout this patent. The correct name of the well-known database located at the Los Alamos National Laboratory is GenBank. Since we are not 100% certain that the latter is intended, we shall retain the apparently incorrect spelling of the author.*

oncogenic power of certain expression products of the adenoviral genome, essentially of the E1 region and probably of the E4 region, at least for certain serotypes. Moreover, the probability of genetic recombination between a defective adenovirus of the prior art, notably a recombinant adenovirus, and either a natural or wild-type adenovirus (originating from an accidental contamination or from an opportunistic infection of a host organism) or an adenoviral genome fragment integrated into the 293 complementation line is not negligible. In fact, one recombination event suffices to restore the E1 function and generate a nondefective recombinant adenovirus capable of being disseminated into the environment. It can also be envisaged that a natural *[or?]* wild-type adenovirus co-infecting the same cell as a defective adenovirus could complement the latter for the E1 function, causing a co-dissemination of both viruses. Finally, certain types of eukaryotic cells produce proteins exhibiting an E1A-like activity also capable of partially complementing the defective adenoviruses which they infect.

Hence it is desirable to have high-performance adenoviral vectors having a minimum of risk with the aim of using them in gene therapy for *in vivo* correction of serious genetic defects and for treatment of certain diseases for which efficacious therapeutic approaches are not available. The success of gene therapy applied to man depends on obtaining them.

Moreover, questions exist concerning the obtaining of the 293 line. These questions can be of a nature to compromise the acceptability of products intended for human use that will be derived from them. It would be useful to have complementation lines whose origin and history are exactly known to produce recombinant adenovirus particles intended for human use.

Now there have been found (1) novel defective adenoviral vectors from which certain specific regions of the adenoviral genome have been deleted and which are more suited to the transfer of an exogenous nucleotide sequence *in vivo*, and (2) novel characterized complementation lines which

are pharmaceutically acceptable and hence offer all the safety characteristics required for the production of products intended for human use.

The benefit of these novel vectors is that they offer an enhanced cloning capacity permitting the insertion of one or more genes of interest of large size and a maximal safety of use. These deleterious mutations make these adenoviruses incapable of autonomous replication and cellular transformation, without altering their capacity for transferring and expressing a gene of interest.

That is why this invention has as its object an adenoviral vector defective for replication, capable of being encapsidated in a complementation cell, which is derived from the genome of an adenovirus comprising, from 5' to 3', a 5' ITR, an encapsidation region, an E1A region, an E1B region, an E2 region, an E3 region, an E4 region and a 3' ITR, by deletion:

(i) of all or part of the E1A region and of the entirety of the part of the E1B region coding for the early proteins; or

(ii) of all or part of the E1A region and all or part of at least one region selected from among the E2 and E4 regions; or

(iii) of all or part of the E1A region and a part of the encapsidation region.

Within the context of this invention, the expression "deletion" or "deprived" refers to the elimination of at least one nucleotide in the targeted region and, of course, it can involve a continuous or discontinuous deletion. By all or part is understood either the entirety or only a part of the considered region. One prefers the deletions that prevent the production of at least one expression product coded by said region. Hence they can be situated in a coding region or in a regulatory region such as the promoter region and can concern at least one nucleotide in a manner such as to destroy the reading

frame of a gene or render a promoter region nonfunctional. They can also involve partial deletions of one or more genes of said region or of the entire region.

An adenoviral vector according to the invention is defective for replication but is capable of being replicated and encapsidated in a complementation cell supplying to it *in trans* the product or products for which it is defective, so as to generate an adenoviral particle (still designated a defective adenovirus) incapable of autonomous replication in a host cell but nevertheless infectious because it has the ability to deliver the vector into a host cell.

According to a first variant, an adenoviral vector according to the invention is derived from the genome of a natural or wild-type adenovirus by deletion of all or part of the E1A region and the part of the E1B region comprising the entirety of the sequences coding for the early proteins. According to a preferred embodiment, it concerns the promoter and the sequences coding for the expression products of the E1B region, i.e., the early proteins, and does not include all or part of the transcription termination signal which covers the sequences coding for the IX late protein. Involving an adenoviral vector according to the invention derived from a type 5 human adenovirus, said deletion comprises at least the sequences included between nucleotides 1634 and 3509 of the adenoviral genome whose sequence is as disclosed in the Genebank database under the reference M73260. The purpose of this deletion is to reduce or eliminate the sequences that are common to an adenoviral vector according to the invention and the adenoviral genome fragment integrated into a complementation line, e.g., the 293 line. Moreover, it eliminates from an adenoviral vector according to the invention the sequences whose expression products are potentially oncogenic, at least in conjunction with the expression products of the E1A region.

In addition, an adenoviral vector according to the invention is derived from the genome of a natural or wild-type adenovirus by deletion of all or part:

- of the E3 region and/or
- of the E2 region and/or
- of the E4 region.

It goes without saying that an adenoviral vector according to the invention comprises one of the three deletions cited above or two of them in any combination whatsoever, or else all of the deletions.

According to a particularly advantageous embodiment, an adenoviral vector according to the invention has deleted from it a part of the E3 region only, and preferably the part which does not contain the sequences coding for the gp19kDa protein. The presence of the sequence coding for the gp19kDa protein in an adenoviral vector according to the invention will enable the infected cells to escape the host's immunosurveillance; an important criterion when the therapeutic protocol necessitates several repeated administrations. One preferably will choose to place the sequences coding for the gp19kDa protein under the control of appropriate elements enabling their expression in the host cell, i.e., the elements necessary for the transcription of said sequences into mRNA and the translation of the latter into protein. In particular, these elements include a promoter. Such promoters are well known by a person skilled in the art and are inserted upstream of said coding sequence by the conventional techniques of gene therapy. The utilized promoter preferably will be a constitutive promoter that cannot be activated by any of the expression products of the E1A region. By way of example, one can cite the promoter of the HMG (Hydroxy-Methyl-Glutaryl coenzyme A reductase) gene, the early promoter of the SV40 virus (Simian Virus 40), the LTR (Long Terminal Repeat) of the RSV (Rous Sarcoma Virus) or the promoter of a PGK (phospho-glycerate kinase) gene of a higher eukaryote.

Moreover, an adenoviral vector according to the invention can optionally have deleted from it the part of the E3 region corresponding to the promoter region, which will be substituted by a heterologous promoter region such as one of those mentioned above.

According to a second variant, an adenoviral vector according to the invention is derived from the genome of a natural or wild-type adenovirus by continuous or discontinuous deletion of all or part of the E1A region and of all or part of at least the E2 and/or E4 region. Such a deletion makes it possible to increase the possibilities for the cloning of genes of interest. Furthermore, eliminating all or part of the E4 region also makes it possible to reduce or eliminate sequences coding for potentially oncogenic products.

As before, an adenoviral vector according to the invention can additionally be deprived of all or part of the E1B and/or E3 regions and, in particular, according to an embodiment such as that mentioned before (such as the deletion of the part of the E1B region comprising the entirety of the sequences coding for the early proteins and of the part of the E3 region not coding for the gp19kDa protein).

Finally, according to a third variant, an adenoviral vector according to the invention is derived from the genome of an adenovirus by deletion of all or part of the E1A region and of a part of the encapsidation region.

A partial deletion of the encapsidation region makes it possible to reduce notably the probability of uncontrolled dissemination of an adenoviral vector according to the invention when the latter is in the presence of a wild-type adenovirus. Such a deletion makes it possible to affect its encapsidation functions in such a way that, even in the event of complementation *in trans* of the latter's defective function by a wild-type adenovirus, it will not be able to be encapsidated efficiently with respect to the genome of the competitive wild-type adenovirus.

The deletions of the encapsidation region will be chosen as a function of 2 criteria: a reduced capacity to be encapsidated, but simultaneously a residual efficiency compatible with industrial production. In other words, the encapsidation function of an adenoviral vector according to the invention is

substantially maintained, although to a lesser degree. The attenuation can be determined by the conventional techniques of titration by infecting an adequate line and evaluating the number of lysis plaques. Such techniques are known by a person skilled in the art. Within the context of the invention, the encapsidation efficiency is reduced by a factor of 2 to 50, advantageously 3 to 20 and preferably 5 to 10 with respect to a control adenovirus having a wild-type encapsidation region.

Of course, an attenuated adenoviral vector according to the invention can additionally comprise at least one or any combination of the deletions cited above.

An adenoviral vector according to this invention is derived from the genome of a natural or wild-type adenovirus, advantageously from a canine, avian or human adenovirus, preferably from a human adenovirus of type 2, 3, 4, 5 or 7 and, in an entirely preferred manner, from a human adenovirus of type 5 (Ad5). In the latter case, the deletions of the adenoviral vector according to the invention are indicated by reference to the position of the nucleotides of the Ad5 genome specified in the Genebank database under the reference M73620.

Particularly preferred is an adenoviral vector according to the invention derived from the genome of a type 5 human adenovirus by deletion:

(i) of the entirety of the part coding for the early proteins of the E1B region and extending from nucleotide 1634 and terminating in nucleotide 4047; and/or

(ii) of the E4 region extending from nucleotide 32800 to nucleotide 35826; and/or

(iii) of the part of the E3 region extending from nucleotide 27871 to nucleotide 30748; and/or

(iv) of the part of the encapsidation region:

- extending from nucleotide 270 to nucleotide 346, or

- extending from nucleotide 184 to nucleotide 273, or

- extending from nucleotide 287 to nucleotide 358.

Preferably, an adenoviral vector according to the invention is derived from the genome of a wild-type or natural adenovirus by deletion of at least 18%, at least 22%, at least 25%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or else at least 95% and notably 98.5% of said genome.

According to a particularly preferred embodiment, an adenoviral vector according to the invention is derived from the genome of an adenovirus by deletion of the entire adenoviral genome except for the 5' and 3' ITRs and all or part of the encapsidation region. According to this variant, it comprises only the minimum of viral sequences in order to limit the risks of recombination, limit the risks of oncogenicity and have a maximal cloning capacity. One will then speak of a "minimum" adenoviral vector in which it will then be possible to insert up to 30 kb of exogenous nucleotide sequence. A preferred adenoviral vector according to the invention is derived from a type 5 human adenovirus by deletion of the part of the viral genome extending from nucleotide 459 to nucleotide 35832.

Within the context of this invention, an adenoviral vector according to the invention has as its object the transfer of an exogenous nucleotide sequence into a host cell and its expression therein. An "exogenous nucleotide sequence" is understood to be a nucleic acid which comprises coding sequences and regulatory sequences making possible the expression of said coding sequences and in which the coding sequences are sequences that are

not normally present in the genome of an adenovirus. The regulatory sequences can be of any origin. The exogenous nucleotide sequence is introduced into an adenoviral vector according to the invention by the standard techniques of genetic engineering, between the encapsidation region and the 3' ITR.

An exogenous nucleotide sequence can be composed of one or more genes of interest and, in a preferred manner, of therapeutic interest. Within the context of this invention, a gene of interest can code either for an antisense RNA or for an mRNA which will then be translated into protein of interest. A gene of interest can be of genomic type, of complementary DNA type (cDNA) or of mixed type (minigene, in which at least one intron is deleted). It can code for a mature protein, a precursor of a mature protein, notably a precursor intended to be secreted and comprising for that reason a signal peptide, a chimeric protein originating from the fusion of sequences of diverse origin, or a mutant of a natural protein exhibiting improved or modified biological properties. Such a mutant can be obtained by mutation, deletion, substitution and/or addition of one or more nucleotides of the gene coding for the natural protein.

A gene of interest can be placed under the control of appropriate elements for its expression in a host cell. By "appropriate elements" is understood all elements necessary for its transcription into RNA (antisense RNA or mRNA) and for the translation of an mRNA into protein. Among the elements necessary for transcription, the promoter is of particular importance. It can be a constitutive promoter or a regulatable promoter and it can be isolated from any gene of eukaryotic or viral origin and even of adenoviral origin. Alternatively, it can be the natural promoter of the gene of interest in question. In general, a promoter used in this invention can be modified so as to contain regulatory sequences. By way of example, a gene of interest in use in this invention is placed under the control of the promoter of immunoglobulin genes when one seeks to target its transfer into lymphocyte host cells. One can also cite the promoter of the HSV-1 TK (type 1 herpes

virus thymidine kinase) gene or else the MLP adenoviral promoter, notably of the type 2 human adenovirus, permitting expression in a large number of cell types.

Among the genes of interest that can be used within the context of this invention, one can cite:

- the genes coding for cytokines, such as alpha interferon, gamma interferon, interleukins;

- the genes coding for membrane receptors, such as the receptors recognized by pathogenic organisms (viruses, bacteria or parasites), preferably by the HIV virus (Human Immunodeficiency Virus);

- the genes coding for coagulation factors, such as factor VIII and factor IX;

- the gene coding for dystrophin;

- the gene coding for insulin;

- the genes coding for proteins participating directly or indirectly in the cellular ion channels, such as the CFTR (Cystic Fibrosis Transmembrane Conductance Regulator) protein;

- the genes coding for antisense RNAs or proteins capable of inhibiting the activity of a protein produced by a pathogenic gene present in the genome of a pathogenic organism or by a cellular gene whose expression is deregulated, e.g., an oncogene;

- the genes coding for a protein inhibiting an enzymatic activity, such as α_1-antitrypsin[2] or an inhibitor of a viral protease;

- the genes coding for variants of pathogenic proteins that have been mutated so as to alter their biological function, such as, for example, trans-dominant variants of the TAT protein of the HIV virus capable of competing with the natural protein for the bond to the target sequence, thereby preventing the activation of the HIV;

- the genes coding for antigenic epitopes in order to increase the immunity of the host cell;

- the genes coding for class I and II major histocompatibility complex proteins, as well as the genes coding for the inducer proteins of those genes;

- the genes coding for cellular enzymes or [enzymes] produced by pathogenic organisms; and

- the suicide genes. One can cite more particularly the HSV-1 TK suicide gene. The viral TK enzyme exhibits a clearly superior affinity compared to the cellular TK enzyme for certain nucleoside analogs (such as acyclovir or ganciclovir[3]). It converts them into monophosphated molecules which are themselves convertible by cellular enzymes into nucleotide precursors which are toxic. These nucleotide precursors can be incorporated into DNA molecules in the course of being synthesized, hence primarily into the DNA of cells in a state of

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[2] *Tr. note: Written incorrectly as α1-antitrypsin in the French original.*

[3] *Tr. note: Misspelled as gancyclovir in the French text. We shall use the correct spelling throughout.*

replication. This incorporation makes possible the specific destruction of dividing cells such as cancer cells.

This list is not limiting, and other genes of interest can be used within the context of this invention.

Furthermore, according to another embodiment of the invention, an adenoviral vector according to the invention can additionally comprise a nontherapeutic gene coding for a nonadenoviral transcription trans-activating protein. Of course, one will avoid the gene or genes of the E1A region coding for a trans-activating protein, whose expression would risk making the adenovirus nondefective. One preferably will choose the gene coding for the Gal4 protein of *Saccharomyces cerevisiae*. Its expression will make possible the propagation of the vector in a complementation line such as the one described hereinbelow. Such a line is more sophisticated and makes it possible to mitigate any toxicity problems due to the continuous production of complementation adenoviral proteins. The gene coding for a transcription trans-activating protein can be placed, if necessary, under the control of elements appropriate for its expression, e.g., those which make possible the expression of a gene of interest.

The invention also concerns an adenoviral particle and a eukaryotic host cell comprising an adenoviral vector according to the invention. Said cell is advantageously a mammalian cell and preferably a human cell and can include said vector in a form integrated into the genome or, preferably, in nonintegrated form (episome).

An adenoviral particle according to the invention can be prepared by transfer into any complementation line supplying *in trans* the functions for which an adenoviral vector according to the invention is defective, e.g., the 293 line of the prior art. These preparation techniques are known by a person skilled in the art (Graham and Prevec, 1991, Methods in Molecular Biology, vol. 7, 109-128, Ed.: E.J. Murey, The Human Press Inc.). Optionally, an adenoviral

particle according to the invention can be generated in a complementation line according to the invention such as that described hereinbelow.

That is why this invention also concerns a complementation line comprising a complementation element, comprising notably a part of the E1 region of the genome of an adenovirus, excluding the 5' ITR; said complementation element being capable of complementing *in trans* a defective adenoviral vector and being integrated into the genome of said complementation line or inserted into an expression vector.

Within the scope of this invention, the term "complementation line" refers to a eukaryotic cell capable of supplying *in trans* the function or functions for which an adenoviral vector is defective. In other words, it is capable of producing the protein or proteins that are necessary for the replication and encapsidation of said adenoviral vector, early and/or late proteins which it cannot produce itself and which are necessary for the construction of a viral particle. Of course, said part can be modified by mutation, deletion and/or addition of nucleotides, since such modifications do not alter its complementation capacity. Thus, an adenoviral vector which is defective for the E1 function will have to be propagated in a complementation line for E1 (capable of supplying *in trans* the protein or all the proteins coded by the E1 region which the vector cannot produce), a vector which is defective for the E1 and E4 functions will have to be [propagated] in a complementation line for E1 and E4 (supplying the necessary proteins coded by the E1 and E4 regions) and, finally, a vector which is defective for the E1, E2 and E4 functions will have to be [propagated] in a complementation line for the three functions. As indicated in the introduction, the E3 region is nonessential and does not have to be specifically complemented.

A complementation line according to the invention can be derived either from an immortalized cell line capable of dividing indefinitely or from a primary line. In conformance with the aims of this invention, a complementation line according to the invention is usable for the encapsidation of any defective

adenoviral vector and, in particular, of an adenoviral vector according to the invention. Thus, when the term "defective adenoviral vector" is used hereinbelow, it should be understood that it makes reference to any defective vector whatsoever, whether of the prior art or of this invention.

By "complementation element" is understood a nucleic acid comprising at least the part of the adenoviral genome in use within the context of this invention. It can be inserted on a vector, for example of the plasmid or viral type, for example retroviral, adenoviral or derived from a poxvirus. Nevertheless, one will prefer the case in which it is integrated into the genome of a complementation line according to the invention. The methods for introducing a vector or a nucleic acid into a cell line and possibly integrating it into the genome of a cell constitute conventional techniques which are well known by a person skilled in the art, as are the vectors usable for such purposes. The complementation element can be introduced into a complementation line according to the invention, either prior to or concomitant with a defective adenoviral vector.

According to a specific embodiment, a complementation line according to the invention is intended to complement *in trans* an adenoviral vector which is defective for the E1 function. Such a line offers the advantage of diminishing the risks of recombination since, in contrast to the conventional 293 line, it is deprived of the 5' ITR present in the vectors.

Within the scope of this invention, a complementation line according to the invention can comprise all or part of the E1A region of the genome of an adenovirus and:

(i) all or part of at least one region of the adenoviral genome selected from among the E1B, E2 and E4 regions, or

(ii) all or part of at least two of the E1B, E2 and E4 regions of said genome, or

(iii) all or part of the E1B, E2 and E4 regions of said genome.

Within the scope of this invention, said regions can be placed, if necessary, under the control of appropriate elements permitting their expression, but it is preferable to place them under the control of their own promoter, inducible by the transcription trans-activating protein coded by the E1A region.

By way of indication, a complementation line according to variant (ii) comprising the E1A, E1B and E4 regions is intended for the preparation of an adenovirus which is defective for the E1 and E4 regions, from which all or part of the corresponding regions have been deleted.

According to an advantageous embodiment, a complementation line according to the invention comprises notably all or part of the E1A region and the entirety of the sequences coding for the early proteins of the E1B region.

Furthermore, according to a variant of this embodiment, a complementation line according to the invention can additionally be deprived of the promoter region of the E1A region. In that case, the part of the adenoviral genome coding for the early proteins of said E1A region will be placed under the control of an appropriate heterologous promoter which is functional in said complementation line. It can be isolated from any eukaryotic or viral gene. However, one will avoid having recourse to an adenoviral promoter of an early region. It can be a constitutive promoter. By way of example, one can cite the promoters of the SV40 virus, of the HSV-1 TK gene and of the murine PGK gene.

Alternatively, the promoter used can be regulatable and, advantageously, inducible by a nonadenoviral transcription trans-activating protein. It can be a promoter isolated from a naturally inducible gene or any promoter modified by the addition of activation sequences (or UAS, for Upstream Activating

Sequences in English) responding to said trans-activating protein. More particularly, it is preferable to use a promoter inducible by the Gal4 protein of *Saccharomyces cerevisiae* and, preferably, a hybrid promoter consisting of a so-called "minimum" promoter containing only the transcription initiation sequences (TATA box and initiation site) of any gene (for example, of the HSV-1 TK gene or MLP of Ad2), upstream of which has been inserted at least one sequence for activation of the Gal10 gene of *Saccharomyces cerevisiae* (Webster et al., 1988, Cell, *52*, 169-178). The latter can be synthesized chemically or isolated from the Gal10 gene by the standard techniques of genetic engineering. Thus the hybrid promoter will be activated and induce expression of the genes coded by the E1A region placed under its control only in the presence of the Gal4 protein. Then the expression products of the E1A region will in turn be able to induce the expression of other early regions, E1B, E2 and/or E4, that may be contained in a complementation line according to the invention. This specific embodiment of the invention avoids the production in a constitutive (possibly toxic) manner of adenoviral proteins that are necessary for the complementation. Hence the induction can be triggered in the presence of an adenoviral vector according to the invention expressing the Gal4 protein. However, such a line can also be used to prepare any defective adenoviral vector, but on the condition that the Gal4 protein is supplied *in trans*. The means for supplying a protein *in trans* are known by a person skilled in the art.

In general, a complementation line comprises a part of the genome of an adenovirus which advantageously is derived from an animal adenovirus, such as a canine or avian adenovirus, or preferably from a human adenovirus, most particularly of type 2 or 5.

A complementation line according to the invention notably comprises the part of the genome of a type 5 human adenovirus extending:

(i) from nucleotide 100 to nucleotide 5297 of the sequence as disclosed in the Genebank database under reference M73260, or

(ii) from nucleotide 100 to nucleotide 4034, or

(iii) from nucleotide 505 to nucleotide 4034.

Advantageously, the part of the genome according to (ii) is inserted upstream of a transcription termination signal, such as for example the polyadenylation signal of the SV40 virus (Simian Virus 40) or of the rabbit β-globin gene; whereas the part according to (iii), which comprises neither the promoter sequences of the E1A region nor the transcription termination signal of the E1B region, is placed under the control of an appropriate promoter, notably a promoter inducible by the Gal4 protein, and of a transcription termination signal, for example that of the rabbit β-globin gene. Such a complementation line is considered to be particularly safe, since it is deprived of a majority of the sequences that are common to [those of] a defective adenovirus.

Furthermore, a complementation line according to the invention can comprise the part of the E4 region of a type 5 human adenovirus starting from nucleotide 32800 and terminating at nucleotide 35826 of the sequence as disclosed in the Genebank database under reference M73260.

In addition, a complementation line according to the invention can comprise the entire genome of a natural adenovirus except for the encapsidation region and the 5' and 3' ITRs and, in an entirely preferred manner, the part of the genome of a type 5 human adenovirus starting from nucleotide 505 and terminating at nucleotide 35826 of the sequence as disclosed in the Genebank database under reference M73260. For the purposes of this invention, the latter is placed under the control of an appropriate promoter. One preferably will have recourse to a promoter that is inducible by the Gal4 protein of *Saccharomyces cerevisiae*. Such a line will make it possible to complement

in trans all the functions that are essential for the replication and encapsidation of an adenoviral vector which is defective for the E1, E2 and E4 functions, notably of a minimum adenoviral vector according to the invention.

According to a preferred embodiment, a complementation line according to the invention can comprise a complementation element comprising, in addition, a gene coding for a selection marker permitting the detection and isolation of cells containing it. Within the context of this invention, it can be any gene coding for a selection marker, these generally being known by a person skilled in the art, advantageously a gene of resistance to an antibiotic and preferably the gene coding for puromycin acetyl-transferase (pac gene) conferring resistance to puromycin.

Within the scope of this invention, the gene coding for a selection marker can be placed under the control of appropriate elements permitting its expression. It can be a constitutive promoter such as the early promoter of the SV40 virus. However, one will prefer a promoter inducible by the trans-activating protein coded by the E1A region, in particular the E2A adenoviral promoter. Such a combination will introduce a selection pressure to maintain the expression of the genes of the E1A region in a complementation line according to the invention. For the purposes of this invention, the promoter used can be modified by deletion, mutation, substitution and/or addition of nucleotides.

According to an entirely preferred embodiment, a complementation line according to the invention is derived from a pharmaceutically acceptable cell line. By "pharmaceutically acceptable cell line" is understood a characterized cell line (whose origin and history are known) and/or a cell line that has already been used for the large-scale production of products intended for human use (constitution of lots for advanced clinical tests or lots intended for sale). Such lines are available in organizations such as the ATCC. In that regard, one can mention the Vero African green monkey kidney line, the

BHK golden or Syrian hamster kidney line, the human line derived from an A549 lung carcinoma, the MRC5 human pulmonary line, the W138 human pulmonary line and the CHO Chinese hamster ovary line.

Alternatively, a complementation line according to the invention can be derived from primary cells, and notably from retina cells taken from a human embryo.

The invention also concerns a process for preparation of an adenoviral particle according to the invention, according to which:

- one introduces an adenoviral vector according to the invention into a complementation line capable of complementing *in trans* said vector, so as to obtain a transfected complementation line,

- one cultures said complementation line under appropriate conditions to make possible the production of said adenoviral particle, and

- one recovers said particle in the cell culture.

Of course, the adenoviral particle can be recovered from the culture supernatant but also from cells according to conventional protocols.

Preferably, a process according to the invention makes use of a complementation line according to the invention.

The invention also has as an object the therapeutic or prophylactic use of an adenoviral vector, an adenoviral particle, a eukaryotic host cell or a complementation line according to the invention.

Finally, this invention relates to a pharmaceutical composition comprising as therapeutic or prophylactic agent an adenoviral vector, an adenoviral particle,

a eukaryotic cell or a complementation cell according to the invention, in association with a pharmaceutically acceptable support medium.

The composition according to the invention is intended in particular for the preventive or curative treatment of diseases such as:

- genetic diseases such as hemophilia, mucoviscidosis or dystrophy of Duchenne or Becker,

- cancers such as those induced by oncogenes or viruses,

- retroviral diseases such as AIDS (acquired immunodeficiency syndrome resulting from infection by HIV), and

- recurrent viral diseases such as viral infections caused by the herpes virus.

A pharmaceutical composition according to the invention can be fabricated in a conventional manner. In particular, one associates a therapeutically efficacious quantity of a therapeutic or prophylactic agent with a support medium such as a diluent. A composition according to the invention can be administered by aerosol or by any conventional pathway in use in the field of the art, in particular by oral, subcutaneous, intramuscular, intravenous, intraperitoneal, intrapulmonary or intratracheal pathway. The administration can take place in a single dose or in doses repeated one or more times after a certain interval. The appropriate administration pathway and dosage vary as a function of various parameters, for example of the treated individual or of the disease to be treated or of the gene or genes of interest to be transferred. In general, a pharmaceutical composition according to the invention comprises a dose of adenovirus according to the invention of between 10^4 and 10^{14}, advantageously between 10^5 and 10^{13}, and preferably between 10^6 and 10^{11}. A pharmaceutical composition, in particular for prophylactic purposes, can additionally comprise a pharmaceutically acceptable adjuvant.

The invention also extends to a treatment method according to which one administers a therapeutically efficacious quantity of an adenoviral vector, adenoviral particle, eukaryotic cell or complementation line according to the invention to a patient having need for such a treatment.

This invention is described more completely with reference to the following Figures and by means of the following examples.

Figure 1 is a diagrammatic representation of the genome of the type 5 human adenovirus (represented in arbitrary units from 0 to 100), indicating the location of various genes.

Figure 2 is a diagrammatic representation of the pTG6546 vector.

Figure 3 is a diagrammatic representation of the pTG6581 vector.

Figure 4 is a diagrammatic representation of the pTG6303 vector.

Figure 5 is a diagrammatic representation of the pTG1660 and pTG1661 vectors.

Figure 6 is a diagrammatic representation of the pTG1653, pTG1654 and pTG1655 vectors.

Figure 7 is a diagrammatic representation of the pTG5913 vector.

Figure 8 is a diagrammatic representation of the pTG8512 vector.

Figure 9 is a diagrammatic representation of the pTG8513 vector.

Figure 10 is a diagrammatic representation of the pTG8514 vector.

Figure 11 is a diagrammatic representation of the pTG8515 vector.

EXAMPLES

The following examples illustrate only one embodiment of this invention.

The constructions described hereinbelow are realized according to the general techniques of genetic engineering and molecular cloning as described in detail in Maniatis et al. (1989, Laboratory Manual, Cold Spring Harbor, Laboratory Press, Cold Spring Harbor, NY). All cloning steps using bacterial plasmids are realized by transfer into the 5K or BJ *Escherichia coli (E. coli)* strain, while those using vectors derived from the M13 phage are realized by transfer into NM 522 *E. coli*. As concerns the PCR amplification steps, one applies the protocol as described in PCR Protocols -A Guide to Methods and Applications (1990, edited by Innis, Gelfand, Sninsky and White, Academic Press Inc.).

Furthermore, the cells are transfected according to the standard techniques which are well known by a person skilled in the art. One can cite the calcium phosphate technique (Maniatis et al., *supra*). But other protocols making it possible to introduce a nucleic acid into a cell can also be used, such as the DEAE dextran technique, electroporation, methods based on osmotic shocks, microinjection of a selected cell or methods based on the use of liposomes.

The inserted fragments in the various constructions described hereinbelow are indicated precisely according to their position in the nucleotide sequence:

- of the Ad5 genome as disclosed in the Genebank database under reference M73260,

- of the type 2 adenovirus (Ad2) genome as disclosed in the Genebank database under reference J01949,

- of the SV40 virus genome as disclosed in the Genebank database under reference J02400.

EXAMPLE 1: Generation of an "attenuated" adenovirus comprising a deletion of a part of the encapsidation region

1. Construction of an "attenuated" vector comprising a deletion from nucleotide 184 to nucleotide 273 of the encapsidation region

One constructs a vector comprising

- the 5' ITR of the Ad5 genome (from nucleotide 1 to nucleotide 103),

- the Ad5 encapsidation region included between the nucleotides from 104 to 458 wherein the portion extending from nucleotide 184 to nucleotide 273 is deleted and the thymine (T) at position 176 is modified into a cytosine (C) in order to create an *Aat*II restriction site,

- an expression cassette of a gene of interest comprising, from 5' toward 3', the MLP of Ad2 (nucleotides 5779 to 6038), the *Kpn*I-*Xba*I-*Hind*III and *Bam*HI restriction sites, the human cDNA coding for the CFTR protein, (the amino acid composition corresponds to the sequence published by Riordan et al., 1989, Science, *245*, 1066-1073, with the exception of a valine in place of the methionine at position 470), the *Pst*I, *Xho*I and *Sal*I sites, and finally the transcription termination signal of the SV40 virus (nucleotides 2665 to 2538), and

- the fragment of the Ad5 genome extending from nucleotide 3329 to nucleotide 6241.

First, between the *Eco*RI and *Eco*RV sites of the M13TG131 vector (Kieny et al., 1983, Gene, *26*, 91-99), one clones the *Eco*RI-*Sma*I fragment isolated from pMLP11. This construction is produced from pMLP10 (Levrero et al.,

1991, Gene, *101*, 195-202) and differs from the parent vector by the introduction of an *Sma*I site at the *Hind*III site. One obtains the M13TG6501 vector. The latter is subjected to a directed mutagenesis in order to delete the sequences included between nucleotides 184 and 273 of the encapsidation region. The directed mutagenesis is implemented with a commercial kit (Amersham) according to the supplier's recommendations and makes use of the OTG4174 oligonucleotide carried under the sequence identifier No. 1 (SEQ ID NO: 1). The mutated vector is designated M13TG6502. The encapsidation region thereby deleted is reintroduced in the form of an *Eco*RI-*Bgl*II fragment, the *Bgl*II site being rendered free by treatment with DNA polymerase Klenow fragment, into the pMLP11 vector digested by *Eco*RI and *Sma*I.

The vector obtained, pTG6500, is partially digested by *Pst*I, treated with DNA polymerase of the T4 phage, then digested by *Pvu*I. One inserts into this vector the *Pvu*I-*Hpa*I fragment isolated from pTG5955 (derived from pMLP11). This fragment comprises the transcription termination signal of the SV40 virus and the part of the Ad5 genome extending from nucleotide 3329 to nucleotide 6241. The pTG6505 vector thereby generated is partially digested by *Sph*I, treated with the DNA polymerase of the T4 phage and religated in order to destroy the *Sph*I site situated at 5' of the polylinker. The result is pTG6511 in which, after digestion by *Bam*HI and treatment with DNA polymerase Klenow fragment, one clones human CFTR cDNA in the form of a fragment at the free ends generated by *Xho*I and *Ava*I digestion and treatment with DNA polymerase Klenow fragment. One obtains pTG6525. By way of indication, the CFTR cDNA is isolated from a plasmid of the prior art such as pTG5960 (Dalemans et al., 1991, Nature, *354*, 526-528).

2. *Construction of an "attenuated" vector comprising a deletion from nucleotide 270 to nucleotide 346 of the encapsidation region*

The M13TG6501 vector is subjected to a directed mutagenesis using the OTG4173 oligonucleotide (SEQ ID NO: 2). Then the mutated fragment is

reintroduced into pMLP11, as indicated above, to generate the pTG6501 vector. The latter is digested by *Sph*I, treated with the DNA polymerase of the T4 phage, then by *Pvu*I. One obtains pTG6546 (Figure 2) by cloning of the *Pvu*I-*Kpn*I fragment (the *Kpn*I site having been rendered free) isolated from pTG6525 and comprising human CFTR cDNA.

3. *Construction of an "attenuated" vector comprising a deletion from nucleotide 287 to nucleotide 358 of the encapsidation region*

The M13TG6501 vector is subjected to a directed mutagenesis in order to delete the sequences included between nucleotides 287 and 358 of the encapsidation region and to modify the thymines at positions 275 and 276 into guanines to introduce an *Nco*1 site. The mutagenesis is realized by means of the OTG4191 oligonucleotide (SEQ ID NO: 3) to yield M13TG6-507. The latter is cleaved by *Bgl*II, treated with DNA polymerase Klenow fragment, then digested by *Eco*RI, and one purifies the corresponding mutated fragment which one introduces into pMLP11 digested by *Eco*RI and *Sma*I. One generates pTG6504, from which one isolates the *Sph*I(site made free by treatment with the DNA polymerase of the T4 phage)-*Pvu*I fragment which one inserts between the *Kpn*I (made free by treatment with T4 polymerase) and *Pvu*I sites of pTG6511. One obtains pTG6513, which is treated with *Bam*HI and DNA polymerase Klenow fragment before insertion of the *Ava*I and *Xho*I fragment of pTG5960 to yield pTG6526.

4. *Generation of a defective and attenuated recombinant adenovirus*

The recombinant defective adenoviruses are generated by co-transfection into 293 cells of either pTG6525, pTG6526 or pTG6546 linearized by *Cla*I and of genomic DNA of Ad-dl324 (Thimmappaya et al., 1982, Cell, *31*, 543-551), also digested by *Cla*I, so as to generate a recombinant virus by homologous recombination. After 8 to 10 days, the individual plaques are isolated, amplified in 293 cells, and analyzed by restriction mapping. Viral stocks

(AdTG6525, AdTG6526 and AdTG6546) are constituted and their titer is determined according to conventional techniques.

The AdTG6546 virus is placed in a competitive situation by co-infection with Ad-CFTR (Rosenfeld et al., 1992, Cell, *68*, 143-155), which comprises an encapsidation region of the wild type. One infects the 293 cells with 5 pfu (plaque-forming units) of Ad-CFTR and 5 pfu of AdTG6546 per cell. One isolates in parallel the viral total DNA by the method of Hirt (Gluzman and Van Doren, 1983, J. Virol., *45*, 91-103) and the viral encapsidated DNA after treating the cells with 0.2% deoxydrolate and then with 10 µg/ml of deoxyribonuclease (DNase) I in order to eliminate the unprotected DNAs in the virions. Whereas the quantities of total DNA of AD-CFTR and AdTG6546 are identical, there is approximately 3 times less encapsidated DNA of ADTG6546 than encapsidated DNA of Ad-CFTR.

One measures the level of expression of the CFTR protein in the cellular extracts of 293 cells infected by AdTG6546. The analysis is performed by Western blot according to the technique described in Dalemans et al. (1991, Nature, *supra*), using the MATG1031 monoclonal antibody. But, any other antibody recognizing the antigenic epitopes of the CFTR protein can be used. One reveals a product having an expected molecular mass of approximately 170 kDa. By way of indication, the production level is nearly equivalent to that obtained in the cellular extracts infected by the Ad-CFTR nonattenuated virus.

EXAMPLE 2: Generation of a defective adenovirus from which are deleted the E1A region and the entirety of the sequences coding for the early proteins of the E1B region

1. Obtaining of a recombinant adenovirus for the expression of the CFTR protein (AdTG6581)

Such an adenovirus is generated starting from a pTG6581 plasmid vector comprising, from 5' toward 3':

- the Ad5 5' ITR (from nucleotide 1 to nucleotide 103),
- the Ad5 encapsidation region (from nucleotide 104 to nucleotide 458),
- an exogenous nucleotide sequence comprising an expression cassette, which comprises the following elements:
  * the Ad2 MLP (nucleotides 5779 to 6038), followed by the three tripartite leaders of Ad2 (nucleotides 6039-6079; nucleotides 7101-7175; nucleotides 9637-9712); these leaders are included in order to enhance the translation efficiency of sequences inserted downstream,
  * a polylinker comprising, from 5' toward 3', the *Xba*I, *Hind*III, *Bam*HI, *Eco*RV, *Hpa*I and *Not*I restriction sites usable for the cloning of a gene of interest,
  * a gene of interest, such as the gene coding for the CFTR protein,
  * the transcription termination signal isolated from the SV40 virus (nucleotides 2543 to 2618),
- the portion of the Ad5 adenoviral genome extending from nucleotide 4047 to nucleotide 6241.

The fragment of the Ad5 genome extending from nucleotide 4047 to nucleotide 4614 is amplified by PCR starting from the genomic DNA of Ad5. The PCR reaction makes use of the sense primer OTG5021 (SEQ ID NO: 4), comprising at its 5' end a *Bam*HI site intended to facilitate the later cloning

steps, and the antisense primer OTG5157 (SEQ ID NO: 5). The fragment thereby generated is treated with DNA polymerase Klenow fragment before being cloned at the *Sma*I site of M13mp1[illegible] (Gibco BRL), yielding M13TG6517. The sequence of the fragment generated by PCR is verified by the standard enzyme method (Sanger et al., 1977, Proc. Natl. Acad. Sci. USA, *74*, 5463).

Furthermore, the *Pvu*I-*Sma*I fragment is isolated from pMLP11. It is cloned between the *Pvu*I and *Kpn*I sites of pTG6511 (example 1.1), the *Kpn*I site having been made free by a treatment with DNA polymerase of the T4 phage according to standard methods. One thereby generates the pTG6547 vector.

The latter is digested by the *Sal*I and *Bst*XI enzymes and ligated to two fragments, firstly the *Bam*HI-*Bst*XI fragment purified from M13TG6517 and secondly the *Xho*I-*Bgl*II fragment from pTG6[illegible]5. The latter comprises notably the transcription termination signal of the SV40 virus framed by the *Xho*I and *Bgl*II restriction sites. However, any other plasmid comprising the same termination sequence and adequate restriction sites could be used. One obtains the pTG6555 vector into which one inserts at the *Bam*HI unique site an adaptor containing two restriction sites generating free ends, *Eco*RV and *Hpa*I. This adaptor originates from the reassociation of the OTG5564 and OTG5565 oligonucleotides (SEQ ID NO: 6 and [illegible]). One obtains pTG6580. Finally, the *Sac*I-*Pst*I fragment of pTG6525, whose ends have been made free and which comprises human CFTR cDNA, is cloned at the *Eco*RV site of pTG6580. One generates pTG6581 (Figure 3).

The corresponding recombinant adenovirus AdTG6581 is generated by co-transfection of pTG6581 and Ad dl324 cleaved by *Cla*I into a complementation line for the E1 function, such as the 293 line or a line from example 6, according to the standard protocol.

2. *Obtaining of a recombinant adenovirus for the expression of IFNγ*

The pTG6303 vector (Figure 4) is obtained by cloning the *Hpa*I-*Sma*I fragment of M13TG2437 at the *Hpa*I site of pTG6580. The latter originates from the cloning in an M13TG130 vector (Kieny et al., 1983, *supra*) of the gene coding for gamma interferon (IFNγ), whose sequence is as specified in Gray et al. (1982, Nature, *295*, 503-508). The AdTG6303 recombinant adenovirus is obtained according to standard techniques by homologous recombination resulting from the co-transfection of pTG6303 and Ad dl324 linearized by *Cla*I into a complementation line for the E1 function.

3. *Construction of an adenovirus from which the E1 region is deleted and in which the E3 region is placed under the control of a constitutive promoter*

The pTG1670 vector is obtained by cloning, between the *Aat*II and *Bam*HI sites of the p polyII vector (Lathe et al., 1987, Gene, *57*, 193-201), of a PCR fragment comprising the 3' LTR (Long Terminal Repeat) of the RSV (Rous Sarcoma Virus) virus. The PCR reaction makes use of the pRSV/L vector (De Wet et al., 1987, Mol. Cell. Biol., *7*, 725-737) as matrix and the OTG5892 and OTG5893 primers (SEQ ID NO: 8 and 9).

Furthermore, the 5' part of the E3 region (nucleotides 27588 to 28607) is amplified by PCR starting from the pTG1659 vector and with the aid of the OTG5920 and OTG5891 primers (SEQ ID NO: 10 and 11). The latter is constructed in several steps. The *Bam*HI-*Avr*II fragment (nucleotides 21562 to 28752) is obtained from the genomic DNA of Ad5, then is cloned between the same sites of pTG7457 to generate pTG1649. The pTG7457 vector is a pUC19 (Gibco BRL) modified at the polylinker so as to contain notably an *Avr*II site. Then one introduces the *Eco*RI(Klenow)-*Avr*II fragment of M13TG1646 (example 8) into pTG1649 cleaved by *Avr*II-*Nde*I (Klenow), which yields the pTG1651 vector. Finally, pTG1659 is generated by insertion of the *Avr*II fragment (nucleotides 28752 to 35463) purified from the genomic DNA of Ad5 into pTG1651 linearized by *Avr*II. The PCR fragment is integrated between the *Xba*I and *Bam*HI sites of p poly II to yield

pTG1671. Then one inserts at the latter's *AatII* site an *Eco*RV-*Aat*II fragment obtained from pTG1670 to yield pTG1676.

The *Eco*RI fragment of Ad5 corresponding to nucleotides 27331 to 30049 is isolated starting from a genomic DNA preparation and is subcloned in pBluescript-Sk+ (Stratagene) previously cleaved by *Eco*RI. One obtains pTG1669. The latter is mutated (Amersham kit) by introduction of a *Bam*HI site either at position 27867 (mutagenic oligonucleotide OTG6079; SEQ ID NO: 12) or at position 28249 (mutagenic oligonucleotide OTG6080; SEQ ID NO: 13). One obtains pTG1672 and pTG1673, respectively. One isolates from the pTG1676 vector the *Bam*HI-*Bsi*WI fragment comprising the 3' LTR of RSV followed by the 5' part of the E3 region and one inserts it between the *Bam*HI site (position 27331 or 30049) and the *Bsi*W *[sic]* site (position 28390) of the vectors obtained in the preceding step, to generate pTG1977 and pTG1978. Then the *Eco*RI fragment obtained from each of these two vectors is integrated into pTG1679, replacing its wild-type *Eco*RI fragment. One obtains pTG1679-E3+. By way of indication, the pTG1679 vector results from the cloning of the *Bst*EII-*Kpn*I fragment (site made free by treatment with T4 polymerase) of pTG6590 (example 3.1) between the *Bst*EII site and *Bam*HI site (made free by treatment with polymerase Klenow fragment) of pTG6584 (example 3.1).

One generates an adenovirus particle by homologous recombination in a complementation line for the E1 function, between the *Aat*II fragment of pTG1679-E3+ and an adenoviral vector such as Ad dl324 or Ad-RSVβ-gal. The latter contains the gene of β-galactosidase in place of the E1 region (Stratford-Perricaudet et al., 1992, J. Clin. Invest., *90*, 626-630).

EXAMPLE 3: Construction of a recombinant adenoviral vector with improved cloning capacity by partial deletion of the E1 and E3 regions

1. Construction of pTG6590ΔE3

The fragment carrying the part of the Ad5 genome included between nucleotides 27325 and 27871 is amplified by PCR starting from an Ad5 genomic DNA preparation and with the aid of the OTG6064 and OTG6065 primers (SEQ ID NO: 14 and 15). OTG6065 comprises at its 5' end a *Bsm*I site, which is also present in the E3 region (at position 30750).

The amplified fragment is cloned at the *Sma*I site of M13mp18 to yield M13TG6523. The *Eco*RI-*Bsm*I fragment is isolated from the latter to be introduced into the pTG6590 vector cleaved by the same enzymes. One obtains pTG6590Δ3 *[sic]*, which contains the 3' part of the adenoviral genome (between nucleotides 27082 and 35935) from which is deleted the E3 region included between nucleotides 27872 and 30740, whereas pTG6590 has deleted from it a smaller part of the E3 region (position 28592 to position 30470). The pTG6590 vector is obtained as follows: One generates by PCR a fragment extending from nucleotide 35228 to nucleotide 35935 (comprising the 3' ITR), starting from an Ad5 genomic preparation and with the aid of the OTG5481 and OTG5482 primers (SEQ ID NO: 16 and 17). The latter is then cloned at the *Sma*I site of M13mp18 to yield M13TG6519. Moreover, the pTG6584 vector is digested by *Xba*I and then religated in order to eliminate the corresponding fragment of the E3 region. One obtains pTG6589, which is cleaved by *Bam*HI, treated with Klenow and then digested by *Bst*EII. Into the vector thus treated, one introduces the *Eco*RI (Klenow)-*Bst*EII fragment purified from M13TG6519 to generate pTG6590.

By way of indication, the pTG6584 vector is a pUC19 vector (Gibco BRL) which contains the Ad5 sequences extending from the *Spe*I unique site (position 27082) to the beginning of the promoter region of the E4 region (position 35826). It is obtained by digestion of pTG1659 (example 2.3) by *Sal*1 and *Spe*I, treated with DNA polymerase Klenow fragment, and then religation.

2. *Construction of an adenoviral vector from which are deleted the E1 region and the part of the E3 region not expressing the gp19kDa protein*

The part of the E3 region coding for gp19kDa (nucleotides 28731 to 29217) is obtained by PCR starting from an Ad5 genomic DNA preparation, making use of the OTG5455 and OTG5456 primers (SEQ ID NO: 18 and 19). The fragment generated is introduced at the *Sma*I site of M13mp18 to yield M13TG6520. From the latter, one isolates the *Eco*RI-*Xba*I fragment which one clones at the *Aat*II site of pTG1670 (example 2.3), the sites having been made free by treatment with DNA polymerase Klenow fragment. Then, the *Xba*I fragment purified from the vector of the preceding step is inserted at the *Xba*I site of the pTG6590ΔE3 vector (example 3.1).

3. *Obtaining of adenoviral particles*

The recombinant adenoviral particles are obtained by ligation of the *Spe*I fragments isolated from the genomic DNA of AdTG6303 or AdTG6581 and one or another of the vectors from examples 3.1 and 3.2. Then, the ligation mixture is transfected into a complementation line for the E1 function.

EXAMPLE 4: Construction of an adenovirus from which the E1 and E4 regions are deleted

One amplifies the parts of the adenoviral genome extending from nucleotide 31803 to nucleotide 32799 and from nucleotide 35827 to nucleotide 35935, starting from an Ad5 genomic DNA preparation and the OTG5728 and OTG5729 primers (SEQ ID NO: 20 and 21) and the OTG5730 and OTG5481 primers (SEQ ID NO: 22 and 16), respectively. After a dozen amplification cycles, the reaction is carried on starting from an aliquot of the two reaction mixtures, making use of the OTG5728 and OTG5731 oligonucleotides. The amplified fragment extends from nucleotide 31803 to nucleotide 35935 with a deletion of the entirety of the E4 region (positions 32800 to 35826). After

digestion by *Eco*RI and *Hind*III, it is cloned between the same sites of M13mp18 to yield M13TG6521.

M13 TG6521 is digested by *Eco*RI, treated with DNA polymerase Klenow fragment, then cleaved by *Bst*XI. The 0.46 kb fragment comprising the 3' ITR is inserted between the *Bam*HI site made frec by treatment with DNA polymerase Klenow fragment and the *Bst*XI site of pTG6584 (example 3.1). One obtains pTG6587, which is digested by *Xba*I and then religated onto itself to yield pTG6588 (deletion of E3).

One introduces at the *Pac*I site of pTG6588 a synthetic DNA fragment originating from the reassociation of the OTG6060, OTG6061, OTG6062 and OTG6063 oligonucleotides (SEQ ID NO: 23 to 26). This results in pTG8500 in which the transcription termination signals of the L5 late genes are improved.

One generates an adenoviral particle (AdΔE4) from whose genome is deleted the entirety of the E4 region (nucleotides 32800 to 35826) and the *Xba*I fragment of the E3 region (nucleotides 28592 to 30470), by ligation of the *Spe*I fragments isolated from pTG8500 or pTG6588 or Ad5. The ligation mixture is transfected into a cell complementation line for the E4 function, for example the W162 line (Weinberg and Ketner, 1983, Proc. Natl. Acad. Sci. USA, *80*, 5383-5386). One obtains an adenovirus which is defective for the E1 and E4 functions (ΔE1, ΔE4) by transfection, into a complementation line for E1 and E4 (for example the line of example 8), of the ligation mixture between the genome of Ad dl324 and the pTG8500 or pTG6588 plasmid linearized by *Spe*I.

Furthermore, one can also proceed as follows: One clones the *Spe*I-*Sca*I fragment isolated from pTG1659 (example 2.3) into the pTG6588 vector cleaved by the same enzymes, so as to obtain pTG6591. The latter comprises the Ad5 sequences from nucleotide 21062 to nucleotide 35935 but, as before, with deletion of the entirety of the E4 region and of the *Xba*I fragment of the

E3 region. One introduces the synthetic DNA fragment described above into the pTG6591 vector digested by *Pac*I and one generates pTG6597. The adenoviral particles can be obtained by homologous recombination between the genomic DNA of Ad dl324 cleaved by *Spe*I and the pTG6591 or pTG6597 plasmids cleaved by *Bam*HI.

EXAMPLE 5: Construction of a "minimum" virus

A so-called "minimum" adenoviral vector is constituted by cloning the following elements in a plasmid:

- the Ad5 5' ITR (from nucleotide 1 to nucleotide 103),
- the Ad5 encapsidation region (from nucleotide 104 to nucleotide 458),
- an exogenous nucleotide sequence comprising:
  * a first gene of therapeutic interest, preferably placed under the control of its own promoter in order to obtain a regulation of expression as close as possible to the natural regulation,
  * a second gene of interest consisting of the HSV-1 TK gene, and
  * optionally, any nucleotide sequences added for reasons of replication or encapsidation efficiency in such a manner that the total size of the genome to be encapsidated is between 30 and 36 kb,
  * the sequences coding for the Gal4 protein of *Saccharomyces cerevisiae* (Laughon and Gesteland, 1984, Mol. Cell. Biol., *4*, 260-267) placed under the control of a functional promoter in a higher eukaryotic cell, and

- the Ad5 3' ITR (from nucleotide 35833 to nucleotide 35935).

The assembling of these various elements is realized according to the standard techniques of molecular biology. Infectious virions comprising such a vector are obtained as described before in a complementation line of example 7.

EXAMPLE 6: Constitution of a complementation cell capable of complementing *in trans* the E1 function

1. Constitution of a complementation cell comprising the E1 region from nucleotide 100 to nucleotide 5297 (pTG6533)

It comprises:

- an expression cassette of the pac gene, which is placed under the control of the early promoter of the SV40 virus (nucleotide 5171 to nucleotide 5243) and comprises at 3' the transcription termination signal of SV40 (nucleotide 2543 to nucleotide 2618). The utilized pac gene corresponds to a fragment extending from nucleotide 252 to nucleotide 905 of the sequence disclosed by Lacalle et al. (1989, Gene, *79*, 375-380) and comprising 4 mutations relative to the published sequence (C at position 305 replaced by A; C at position 367 replaced by T; insertion of a G at position 804; deletion of a G at position 820),

- a fragment of the Ad5 genome extending from nucleotide 100 to nucleotide 5297. This fragment comprises the E1A and E1B regions supplied with their own promoter and with their transcription termination signal as well as a fraction of the E2 region, thus covering the sequences that code for the IX protein. By way of indication, it appears that the 293 line is not capable of producing a functional IX protein.

The construction is realized in several steps as detailed below. The p polyIII-I* vector (Lathe et al., 1987, Gene, *57*, 193-201) is subjected to digestion by the *Acc*I and *Eco*RI enzymes. In the vector thus treated, one clones the *Eco*RI-*Cla*I fragment isolated from the pTG6164 plasmid. One obtains the pTG6528 vector.

The pTG6164 plasmid is produced from pLXSN (Miller D., 1989, Bio/Techniques, *7*, 980) and comprises the pac gene placed under the control of the early promoter of the SV40 virus. Briefly, the *Hind*III-*Kpn*I fragment of pLXSN is introduced into M13TG131 to produce M13TG4194. Into the latter, digested by *Nhe*I and *Kpn*I, one inserts the *Nhe*I-*Kpn*I fragment of pMPSV H2 K IL2R (Takeda et al., 1988, Growth Factors, *1*, 59-66) to produce M13TG4196. The latter is digested by *Hind*III-*Kpn*I and one clones the fragment produced from a *Hind*II digestion and a *Kpn*I partial digestion and purification from pLXSN. One obtains pTG5192. The latter is digested by *Hind*III and partially by *Nhe*I, and one introduces the *Hind*III-*Nhe*I fragment of pBabe Puro (Land et al., 1990, Nucleic Acids Res., *18*, 3587), yielding pTG6164.

The pTG6528 vector is digested by *Pst*I, and one introduces at this site the *Pst*I fragment isolated from pTG6185 (example 2.1), comprising the transcription termination signal of SV40. One obtains pTG6529. The latter is subjected to an *Eco*RI-*Hpa*I digestion and is ligated to two fragments, firstly a *Bsp*EI-*Bcg*I fragment (positions 826 to 5297) purified from Ad5 genomic DNA and secondly a fragment generated by PCR at the *Eco*RI and *Bsp*EI ends to yield pTG6531. The PCR fragment is generated by gene amplification starting from Ad5 genomic DNA and the OTG4564 and OTG4565 primers (carried under SEQ ID NO: 27 and 28). The amplified fragment is digested by the *Eco*RI and *Bsp*EI enzymes and used for ligation as indicated in the preceding paragraph.

The pTG6531 vector comprises the 2 transcription units (that of the E1 region and that of the pac gene) in the same orientation. To prevent interference in the transcription, one places them in a head-to-tail orientation (reversed relative to one another) by treating the pTG6531 with *Bam*HI and religating. The pTG6533 vector corresponds to a clone having a reversed orientation of the two units.

The pTG6533 vector is transfected into a mammalian cell line, for example the Vero line (ATCC, CCL81) or the A549 cell line (ATCC, CCL185) by the calcium phosphate technique. The transfected cells are cultured according to the supplier's recommendations and, 24 hours after the transfection, are placed in selective medium containing puromycin (concentration 6 µg/ml). One selects the resistant clones, in which one evaluates the expression of the genes of the E1 region in order to determine the most productive clone, which will be able to be used as a complementation line for the preparation of an adenovirus which is defective for the E1 function, such as that detailed in example 2.

One analyzes the expression of the sequences coding for the early proteins of the E1 region by Northern blot, using appropriate probes marked with the ^{32}P isotope. The production of proteins coded by the E1A region is detected by immunoprecipitation after marking of the cells with the ^{35}S isotope and with the aid of a commercial antibody (Oncogene Science Inc., reference DP11).

One can also verify the capacity of the expression products of the E1A region to activate the promoter of the E1B region (by analysis of the E1B mRNAs by Northern blot) or to activate the promoter of the E2 region (by assay of the enzymatic activity after transient transfection of a "reporter" plasmid comprising the CAT (Chloramphenicol Acetyl Transferase) gene placed under the control of the E2 promoter).

Finally, one can infect these cells with Ad-RSV-βgal (Stratford-Perricaudet et al., 1992, *supra*) and titrate the virus by the agar technique once a cytopathic

effect is observed. In general, one proceeds as follows: The cells are infected to an MOI (multiplicity of infection) of 10. About 48 hours after infection, the cytopathic effect being visible, the cells are lysed and the β-galactosidase activity is assayed according to the conventional protocol (see, for example, Maniatis et al., 1989, *supra*). The positive clones are reinfected to a lower MOI. 48 hours after infection, the supernatant and the cells are harvested according to standard techniques. One determines the viral titer by the agar method, using 293 cells. The ratio of the obtained titer to the starting titer constitutes the amplification factor.

2. *Construction of a complementation line comprising the E1 region from nucleotide 505 to nucleotide 4034 (pTG6557, pTG6558, pTG6559 and pTG6565)*

The pTG6557, pTG6558 and pTG6559 vectors comprise:

(i) an expression cassette of the pac gene (nucleotides 252 to 905 as before) under the control:

- of the E2A promoter of Ad2 (nucleotides 27341 to 27030) (in pTG6558),

- of the E2A promoter of Ad2 from which are deleted the sequences included between nucleotides 27163 and 27182 (for pTG6557). Such a mutation makes it possible to decrease the base level of the E2A promoter without affecting the inducibility by the trans-activating protein coded by E1A, or

- of the SV40 early promoter for pTG6559.

In all three cases, it also comprises at 3' the transcription termination signal of the SV40 virus (nucleotides 2543 to 2618); and

(ii) an expression cassette comprising the part of the E1 region of Ad5 extending from nucleotide 505 to nucleotide 4034. This portion of the adenoviral genome contains the entirety of the sequences coding for the early proteins of the E1A region, the transcription termination signal of the E1A unit, the E1B promoter (inducible by the trans-activating protein coded by E1A) and the entirety of the sequences coding for the E1B region. It also includes the sequences coding for the IX protein, which straddle the E1B region. However, it is deprived of the promoter of the E1A region and of the transcription termination signal of the E1B and IX transcription units. To enable the expression of the sequences of the E1 region, one introduces, at 5' of the adenoviral fragment, the promoter of the PGK murine gene and, at 3', the transcription termination signal of the rabbit β-globin gene (nucleotides 1542 to 2064 of the sequence disclosed in the Genebank database under reference K03256).

Optionally, one can also introduce any nucleotide sequences, for example, ones isolated from pBR322 (Bolivar et al., 1977, Gene, *2*, 95-113), between the expression cassettes of the pac gene and of the E1 region, in order to prevent any transcriptional interference.

These vectors are constructed in several steps as reported below.

First of all, one amplifies by PCR the part of the Ad5 genome extending from nucleotide 505 to nucleotide 826, starting from a genomic preparation and the primers OTG5013, comprising at 5' a *Pst*I site which is useful for the later cloning steps (SEQ ID NO: 29), and OTG4565, straddling the *Bsp*E1 site[4] (SEQ ID NO: 28). The fragment generated by PCR is treated with DNA polymerase Klenow fragment and is then introduced at the *Sma*I site of M13mp18, yielding M13TG6512. The sequence of the PCR fragment is verified.

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[4] *Tr. note: Heretofore the text has always had BspEI, which agrees with what we find in the biotech literature. From this point on, the text sometimes has BspE1. We shall copy what we see in the original text.*

The pTG6533 vector (example 6.1) is digested by the *Eco*RI and *Bsp*E1 enzymes. The vector thus treated is ligated with, on the one hand, the *Pst*I-*Bsp*E1 fragment isolated from M13TG6512 and, on the other hand, the *Eco*RI-*Pst*I fragment isolated from pKJ-1. The latter comprises the portion of the PGK murine gene promoter situated between nucleotides -524 and -19, whose sequence is reported in Adra et al. (1987, Gene, *60*, 65-74). This step yields pTG6552 and makes it possible to insert the PGK murine gene promoter upstream of the Ad5 E1 region starting at nucleotide 505.

Furthermore, the *Xho*I-*Bam*HI fragment, whose end generated by *Xho*I is made free by treatment with DNA polymerase Klenow fragment, is purified from pBCMG Neo (Karasuyama et al., 1989, J. Exp. Med., *169*, 13-25). This fragment, which comprises the transcription termination signal of the rabbit β-globin gene, is introduced between the *Sma*I and *Bam*HI sites of the p polyII-Sfi/Not-14* vector (Lathe et al., 1987, Gene, *57*, 193-201). The resulting pTG6551 vector is digested by the *Sph*I and *Eco*RV enzymes in order to insert therein an Ad5 genome fragment extending from nucleotide 3665 to nucleotide 4034. This fragment is generated by PCR according to the standard protocol. One uses an Ad5 genomic DNA preparation as matrix and the primers OTG5015, which covers the *Sph*I internal site at position 3665 (SEQ ID NO: 30), and OTG 5014, comprising a *Bgl*II site at 5' (SEQ ID NO: 31).

The PCR fragment is treated with DNA polymerase Klenow fragment before being cloned at the *Sma*I site of M13mp18, generating M13TG6516. After verification of its sequence, the PCR fragment is brought out again by digestion by *Bgl*II, treatment with DNA polymerase Klenow fragment and digestion by *Sph*I. It is inserted between the *Sph*I and *Eco*RV sites of pTG6551, resulting in pTG6554.

Moreover, the pTG6529 vector (example 6.1) is subjected to a digestion by the *Hpa*I and *Hind*III enzymes. One purifies the 2.9 kb fragment comprising

the pac gene followed by the transcription termination signal of the SV40 virus. The latter is ligated to the *Sma*I-*Hind*III fragment isolated from pE2 Lac (Boeuf et al., 1990, Oncogene, *5*, 691-699), which carries the E2A promoter of Ad2. One obtains the pTG6556 vector. Alternatively, it can be ligated to the *Sma*I-*Hind*III fragment isolated from pE2 Lac D9170 (Zajchowski et al., 1985, EMBO J., *4*, 1293-1300), which carries the mutated E2A promoter of Ad2. In this case, one obtains pTG6550.

pTG6556 is digested by the *Eco*RI and *Bam*HI enzymes. One inserts between these sites the *Eco*RI-*Sac*II fragment isolated from pTG6552 and the *Sac*II-*Bam*HI fragment isolated from pTG6554. One obtains the pTG6558 vector. The same step performed in pTG6550 and pTG1643 (example 7.1) generates pTG6557 and pTG6559, respectively.

pTG6557 and pTG6558 are digested by *Eco*RV, unique site situated between the two expression cassettes (pac gene and E1 region). At this site one clones a 1.88-kb *Eco*RV-*Pvu*II fragment isolated from pBR322 (Bolivar et al., *supra*) in order to move apart the two promoters, One generates pTG6564 and pTG6565, respectively.

The pTG6557, pTG6558, pTG6564 and pTG6565 vectors are transfected into the A549 cell line. As before, one selects the clones resistant to puromycin and one verifies the expression of the E1 region. The clones expressing E1 are intended to amplify and propagate adenoviruses that are defective for the E1 function. The production of the E1 expression products is accompanied by a cytotoxic effect, but Southern analysis gives no evidence of any rearrangements of vectors. After infection by Ad-RSV-βgal, several clones are capable of amplifying the virus by a factor greater than 100.

3. *Construction of a complementation cell inducible by the Gal4 protein of Saccharomyces cerevisiae*

As before, these vectors comprise the part of the Ad5 E1 region extending from nucleotide 505 to nucleotide 4034. However, the expression of the sequences of the E1A region is placed under the control of an inducible promoter consisting, firstly, of the Ad2 MLP minimal promoter (TATA box and transcription initiation signal; nucleotides -34 to +33) and, secondly, an activation sequence of the Gal 10 gene activatable by the Gal4 protein. The activation consensus signal of 17 nucleotides (17MX), which corresponds to the Gal4 fixation site, is specified in Webster et al. (1988, Cell, *52*, 169). The transcription termination signal of the rabbit β-globin gene is placed at 3' of the E1B transcription unit.

One synthesizes a first DNA fragment comprising a dimer of the 17MX sequence (SEQ ID NO: 32 and 33) followed by the Ad2 MLP minimal promoter and supplied with a *Sal*1 site at its 5' end and a *Bam*HI site at its 3' end. The *Sal*I site is made free by treatment with DNA polymerase Klenow fragment. In addition, one synthesizes a second DNA fragment comprising a pentamer of the sequence followed by the same promoter and supplied with the *Xba*I and *Bam*HI sites at 5' and 3'. After digestion by *Xba*I, the end is made free by treatment with polymerase Klenow fragment.

Each of these fragments is introduced at the *Bgl*II site of p poly II to generate pTG1656 and pTG1657, respectively. Then one introduces into each of the vectors, previously digested by *Pst*I-*Bam*HI, the following two fragments: the *Pst*I-*Xba*I fragment isolated from pTG6552 (example 6.2) and the *Xba*I-*Bam*HI fragment isolated from pTG6559 (example 6.2). One obtains pTG1660 and pTG1661, respectively (Figure 5).

The A549 cells are co-transfected with pTG1643 (expression vector of the pac gene) and either pTG1660 or pTG1661. The clones are selected for their resistance to puromycin and are studied as indicated before. Approximately 50% of the A549-1660 and A549-1661 clones produce expression products of the E1 region. However, the production is accompanied by a cytotoxic effect, modifying the morphological appearance of the cells.

The integration and nonrearrangement of the plasmids in the cellular genome is verified by Southern. No substantial modification of the integrated plasmids (pTG1643, pTG1660 and pTG1661) can be evidenced in the analyzed producer clones. One can also verify the inducibility of the expression of the sequences coded by the E1A region in the presence of Gal4 (by transformation by a plasmid making possible the constitutive expression of the Gal4 protein).

After the infection of several producer clones by Ad-RSV-Bgal *[sic]* to an MOI of approximately 2, two A549-1160 clones are capable of amplifying the viral stock by a factor greater than 100.

EXAMPLE 7: Constitution of a complementation line for all the functions essential for replication of an adenovirus

One constructs a vector comprising the entire adenoviral genome of Ad5 except for the 5' ITR, the 3' ITR and the encapsidation region.

The pTG6528 vector (example 6.1) is digested by the *Pst*I and *Bgl*II enzymes between which one inserts a DNA fragment synthesized chemically according to the standard protocol and consisting of the OTG5039 and OTG5040 oligonucleotides (SEQ ID NO: 34 and 35). The sequence of the oligonucleotides is designed so as not to reconstitute the *Pst*I cloning site and introduce an *Eco*RV site. One obtains pTG1639, which is linearized by digestion by *Eco*RV and ligated to an *Xba*I-*Bam*HI fragment whose ends are made free by treatment with DNA polymerase Klenow fragment. This fragment carries the transcription termination signal of the SV40 virus. Any plasmid comprising a signal surrounded by adequate restriction sites can be used in this step.

The pTG1640 vector thereby generated is digested by *Bam*HI and *Bgl*II, and the fragment carrying the expression cassette of the pac gene is introduced at

the *Bgl*II site of the pPolyII-Sfi/Not-14* vector. One obtains pTG1641. The latter is linearized by *Not*I and treated with DNA polymerase Klenow fragment. One introduces the 0.276-kb *Bam*HI-*Sal*I fragment isolated from pBR322 (Bolivar et al., *supra*), also treated with DNA polymerase Klenow fragment. This yields pTG1643.

The pTG1643 is linearized by *Xho*I, and one inserts at this site an *Xho*I hybrid fragment comprising a 17MX dimer followed by the minimum promoter of the HSV-1 TK gene (nucleotides 303 to 450 of the sequence disclosed in the Genebank database under reference V00467 and supplemented at 3' by an *Xho*I site). One obtains pTG1647, into which the HSV-1 TK 2x17MX hybrid promoter is inserted in the same orientation as the expression cassette of the pac gene.

This construction, pTG1647, serves as base vector for introducing, between the *Pst*I and *Bam*HI sites, an Ad5 genome fragment extending from nucleotide 505 to nucleotide 35826. First, the pTG1647 is digested by *Pst*I and *Bam*HI and then it is ligated, on the one hand, to the *Pst*I-*Cla*I fragment of pTG6552 (example 6.2) comprising the part of the Ad5 genome from nucleotide 505 to nucleotide 918 and, on the other hand, to the *Cla*I-*Bam*HI fragment (positions 918 to 21562) prepared starting from the genomic DNA of Ad5. The vector thus obtained comprises the 5' part of Ad5 except for the 5' ITR and the encapsidation region.

Furthermore, the 3' part of the Ad5 genome is assembled in the pPolyII-Sfi/Not-14* vector. The latter is linearized by *Bam*HI and one introduces the *Bam*HI-*Avr*II fragment (nucleotides 21562 to 28752) of the Ad5 genome and a PCR fragment corresponding to the nucleotides 35463 to 35826 of Ad5. The latter is generated starting from the genomic DNA of Ad5 and the primers OTG5024 (SEQ ID NO: 36) and OTG5025 (SEQ ID NO: 37) and comprises a *Bam*HI site at 5'. The vector obtained is digested by *Avr*II and one inserts the *Avr*II fragment isolated from the genomic DNA of Ad5 and extending from position 28753 to position 35462.

The *Bam*HI fragment comprising the adenoviral sequences is introduced at the *Bam*HI site of the vector from the preceding step comprising the 5' part of the adenoviral genome deprived of the 5' ITR and the encapsidation region.

A complementation line capable of complementing all the functions of a defective adenovirus is generated by transfection into a cell line, such as A549, according to the protocol described in the preceding examples.

One can also proceed by constructing four vectors comprising the quasi-totality of the adenoviral genome which will be reassembled on a single vector during the final step:

- pTG1665 corresponds to the cloning of the *Bsp*EI fragment (nucleotides 826 to 7269), isolated from an Ad5 genomic DNA preparation, at the *Xma*I site of pPolyII-Sfi/Not-14*;

- pTG1664 is generated by insertion of the *Not*I fragment (nucleotides 6503 to 1504), isolated from an Ad5 genomic DNA preparation, at the *Not*I site of the same vector;

- pTG1662 is obtained by introduction of the *Aat*II fragment (nucleotides 10754 to 23970), isolated from an Ad5 genomic DNA preparation, at the *Aat*I site of p polyII;

- pTG1659 comprising the 3' part of the Ad5 genome (example 2.3).

Then one introduces a fragment comprising an inducible expression system such as the promoter described in example 6.3 or 7 inducible by Gal4 or a promoter of the prior art such as the metallothionein or tetracycline promoter. Such a fragment is placed upstream of the 5' sequences of Ad5 (nucleotides 505 to 918) in the pTG1665 vector digested by *Aat*II and *Cla*I. Finally, into the preceding vector and at the corresponding sites, one clones in succession

the *Not*I fragment of pTG1664, the *Aat*II fragment of pTG1662 and finally the *Bam*HI fragment of pTG1659.

A complementation line is generated by co-transfection of the preceding vector and of pTG1643, and one isolates the clones resistant to puromycin. This line is intended more particularly for amplifying and encapsidating the adenoviral vectors of example 5 which are defective for the E1, E2 and E4 functions and for the late functions.

EXAMPLE 8: Constitution of a complementation line for the E1 and E4 functions

The pTG1647 vector (example 7) is digested by the *Pst*I-*Bam*HI enzymes, and one introduces 3 fragments into the vector thus treated:

- the *Pst*I-*Xba*I fragment of pTG6552 (example 6.2) carrying the Ad5 sequences from nucleotide 505 to nucleotide 1339,

- the *Xba*I-*Sph*I fragment of pTG6552 carrying the Ad5 sequences from nucleotide 1340 to nucleotide 3665, and

- the SphI-*Bam*HI fragment of pTG6554 (example 6.2) carrying the Ad5 sequences from nucleotide 3665 to nucleotide 4034 and a transcription termination signal.

The vector thereby obtained is cut by *Bam*HI, and one introduces the following three fragments at that site:

- a fragment digested by *Bam*HI-*Afl*II, generated by PCR, corresponding to the Ad5 sequence situated between position 32800 and position 33104. One uses Ad5 genomic DNA as matrix and the primers OTG5078 (SEQ ID NO: 38) and OTG5079 (SEQ ID NO: 39),

- the *Afl*II-*Avr*II fragment isolated from Ad5 genomic DNA (nucleotide 33105 to nucleotide 35463),

- the *Avr*II-*Bam*HI fragment generated by PCR with the aid of the OTG5024 and OTG5025 primers (see example 7).

The vector thereby generated is introduced into a cell line according to the protocol described before, so as to constitute a complementation line for the E1 and E4 functions.

Moreover, such a line can also be obtained according to the following protocol:

The E4 region of the Ad5 genome (nucleotide 32800 to nucleotide 35826) is reconstituted in several steps. The part extending from nucleotide 33116 to nucleotide 32800 is synthesized by PCR starting from Ad5 genomic DNA with the pair of primers OTG5078 and OTG5079 (SEQ ID NO: 38 and 39), and then is inserted at the *Eco*RV site of M13TG130 to generate M13TG-1645.

The *Bam*HI-*Afl*II fragment of the latter is engaged in a ligation reaction with the *Afl*II-*Avr*II fragment of Ad5 (nucleotide 33104 to nucleotide 35463) and the pTG7457 vector digested by *Bam*HI and *Avr*II. One obtains pTG1650.

Then one completes the E4 region by obtaining the fragment corresponding to nucleotides 35826 to 35457 by PCR starting from an Ad5 genomic DNA preparation and the OTG5024 and OTG5025 primers (SEQ ID NO: 36 and 37). This is inserted at the *Sma*I site of M13mp18 to yield M13TG1646. The *Avr*II-*Eco*RI fragment is isolated from the latter and is cloned between the *Avr*II and *Eco*RI sites of pTG1650. One obtains pTG1652.

The *Bam*HI fragment comprising the E4 region of Ad5 is isolated from pTG1652 and is cloned at the *Bam*HI site of pTG1643, of pTG6559 (example 6.2) or at the *Ssp*I site of pTG6564 (example 6.2) after the sites have been made free, generating pTG1653, pTG1654 and pTG1655 (Figure 6), respectively.

Using conventional techniques, one generates a complementation cell capable of complementing *in trans* the E1 and E4 functions, by:

(1) transformation of pTG1653 into the 293 cell line, or
(2) transformation of pTG1654 or pTG1655 into the A549 cell line.

In general, the expression of the products of the E1 and E4 regions is accompanied by a cytotoxic effect. A certain number of 293-1653 clones is capable of complementing simultaneously adenoviruses from which E1 is deleted and adenoviruses from which E4 is deleted.

Another alternative consists of proceeding as follows:

The M13TG1646 vector is subjected to a directed mutagenesis with the mutagenic oligonucleotide OTG5991 (SEQ ID NO: 40) with the purpose of deleting the promoter of the E4 region and inserting an *Hpa*I site, The mutated vector is designated M13TG6522. It is digested by *Pst*I, treated with DNA polymerase of the T4 phage, then by *Avr*II and ligated with an *Eco*RI(Klenow)-*Avr*II fragment purified from pTG1652 (example 8) to yield pTG6595. The latter is cleaved by *Hpa*I and one introduces the 0.8-kb fragment obtained from pTG5913 (Figure 7) after digestion by *Bgl*II and *Bam*HI and treatment with Klenow fraction. One generates pTG6596 in which the E4 region (positions 32800 to 35826) is placed under the control of the TK promoter. By way of indication, pTG5913 carries the HSV-1 TK gene, and the *Bgl*II-*Bam*HI fragment corresponds to the promoter of that gene (Wagner et al., 1981, Proc. Natl. Acad. Sci. USA, *78*, 1441-1445).

In parallel, the pTG1643 and pTG6559 vectors are linearized by *Bam*HI and one inserts a synthetic fragment produced from the reassociation of the OTG6141 and OTG6142 oligonucleotides (SEQ ID NO: 41 and 42) to obtain pTG8508 and pTG8507, respectively. The latter are cleaved by *Bam*HI before the introduction of the *Bam*HI fragment purified from pTG6596 comprising the expression cassette of E4. One generates the vectors pTG8512 (Figure 8) and pTG8513 (Figure 9).

Furthermore, the introduction of the *Bam*HI fragment of pTG1652 into the pTG8508 or pTG8507 vector linearized by the same enzyme leads to pTG8514 and pTG8515, respectively (Figures 10 and 11).

The cell lines transfected by pTG8512 or pTG8515 will make it possible to complement an adenovirus which is defective for the E4 function, whereas those resulting from the transfection of pTG8513 or pTG8514 are intended to amplify and propagate adenoviruses which are defective for the E1 and E4 functions. Likewise, the transfection of pTG8512 or pTG8515 into 293 cells will make it possible to complement adenoviruses which are defective for E1 and E4.

Claims

1. An adenoviral vector defective for replication, capable of being encapsidated in a complementation cell, which is derived from the genome of an adenovirus comprising, from 5' to 3', a 5' ITR, an encapsidation region, an E1A region, an E1B region, an E2 region, an E3 region, an E4 region and a 3' ITR, by deletion:

   (i) of all or part of the E1A region, and of the entirety of the part of the E1B region coding for the early proteins; or

   (ii) of all or part of the E1A region and all or part of at least one region selected from among the E2 and E4 regions; or

   (iii) of all or part of the E1A region and a part of the encapsidation region.

2. An adenoviral vector according to claim 1, which is derived from the genome of an adenovirus by deletion of all or part of the E1A region and of the entirety of the part of the E1B region coding for the early proteins.

3. An adenoviral vector according to claim 2, which is derived additionally from the genome of an adenovirus by deletion of all or part of the E3 region.

4. An adenoviral vector according to claim 2 or 3, which is derived additionally from the genome of an adenovirus by deletion of all or part of the E2 region.

5. An adenoviral vector according to one of claims 2 to 4, which is derived additionally from the genome of an adenovirus by deletion of all or part of the E4 region.

6. An adenoviral vector according to claim 1, which is derived from the genome of an adenovirus by deletion of all or part of the E1A region and all or part of the E2 region.

7. An adenoviral vector according to claim 1, which is derived from the genome of an adenovirus by deletion of all or part of the E1A region and all or part of the E4 region.

8. An adenoviral vector according to claim 6 or 7, which is derived from the genome of an adenovirus by deletion of all or part of the E1B region.

9. An adenoviral vector according to one of claims 6 to 8, which is derived additionally from the genome of an adenovirus by deletion of all or part of the E3 region.

10. An adenoviral vector according to claim 6, 8 or 9, which is derived from the genome of an adenovirus by deletion of all or part of the E4 region.

11. An adenoviral vector according to one of claims 3 to 5, 9 or 10. which is derived from the genome of an adenovirus by partial deletion of the E3 region of said genome while maintaining the part of said E3 region coding for the gp19kDa protein.

12. An adenoviral vector according to claim 11, in which the part of the E3 region coding for the gp19kDa protein is placed under the control of elements appropriate for the expression of said protein in the host cell.

13. An adenoviral vector according to one of claims 1 to 12, which is derived from the genome of an adenovirus by deletion of all or part of the E1A region and a part of the encapsidation region.

14. An adenoviral vector according to claim 13, which is derived from the genome of a type 5 human adenovirus by deletion of the part of the encapsidation region:

(i) extending from nucleotide 270 to nucleotide 346;

(ii) extending from nucleotide 184 to nucleotide 273; or

(iii) extending from nucleotide 287 to nucleotide 358.

15. An adenoviral vector according to one of claims 1 to 14, which is derived from the genome of an adenovirus selected from among the canine, avian and human adenoviruses.

16. An adenoviral vector according to claim 15, which is derived from the genome of a type 5 human adenovirus.

17. An adenoviral vector according to claim 16, which is derived from the genome of a type 5 human adenovirus by deletion of the part of the E1B region extending from nucleotide 1634 to nucleotide 4047 at the least.

18. An adenoviral vector according to claim 16 or 17, which is derived from the genome of a type 5 human adenovirus notably by deletion of the part of the E3 region extending from nucleotide 27871 to nucleotide 30748.

19. An adenoviral vector according to one of claims 16 to 18, which is derived from the genome of a type 5 human adenovirus notably by deletion of the part of the E4 region extending from nucleotide 32800 to nucleotide 35826.

20. An adenoviral vector according to one of claims 1 to 19, which is derived from the genome of an adenovirus by deletion of at least 18% of the genome of said virus.

21. An adenoviral vector according to claim 20, which is derived from the genome of an adenovirus by deletion of at least 22% of the genome of said virus.

22. An adenoviral vector according to claim 21, which is derived from the genome of an adenovirus by deletion of at least 40% of the genome of said virus.

23. An adenoviral vector according to claim 22, which is derived from the genome of an adenovirus by deletion of at least 95% of the genome of said virus.

24. An adenoviral vector according to claim 23, which is derived from the genome of an adenovirus by deletion of the entire genome of said adenovirus except for the 5' and 3' ITRs and all or part of the encapsidation region.

25. An adenoviral vector according to claim 24, which is derived from the genome of a type 5 human adenovirus by deletion of the part of the viral genome extending from nucleotide 459 to nucleotide 35832.

26. An adenoviral vector according to one of claims 1 to 25, which comprises additionally an exogenous nucleotide sequence.

27. An adenoviral vector according to claim 26, which comprises additionally a gene of interest placed under the control of elements necessary for its expression.

28. An adenoviral vector according to one of claims 26 or 27, which comprises additionally a gene coding for a nonadenoviral transcription trans-activating protein; said gene being placed under the control of elements necessary for the expression of said protein in a host cell.

29. An adenoviral vector according to claim 28, comprising the gene coding for the *Saccharomyces cerevisiae* Gal4 transcription trans-activating protein.

30. An adenovirus particle comprising an adenoviral vector according to one of claims 1 to 29.

31. A eukaryotic host cell comprising an adenoviral vector according to one of claims 1 to 29 or an adenovirus particle according to claim 30.

32. A complementation line comprising a complementation element, comprising notably a part of the E1 region of the genome of an adenovirus, excluding the 5' ITR; said complementation element being capable of complementing *in trans* a defective adenoviral vector and being integrated into the genome of

said complementation line or inserted into an expression vector.

33. A complementation line according to claim 32, comprising notably:

(i) all or part of the E1A region of the genome of an adenovirus; and

(ii) all or part of at least one region of said genome selected from among the E1B, E2 and E4 regions.

34. A complementation line according to claim 32, comprising notably:

(i) all or part of the E1A region of the genome of an adenovirus; and

(ii) all or part of at least two of the E1B, E2 and E4 regions of said genome.

35. A complementation line according to claim 32, comprising notably:

(i) all or part of the E1A region of the genome of an adenovirus; and

(ii) all or part of the E1B, E2 and E4 regions of said genome.

36. A complementation line according to one of claims 33 to 35, comprising notably all or part of the E1A region and the entirety of the part of the E1B region of the genome coding for the early proteins.

37. A complementation line according to one of claims 32 to 36, comprising notably a part of the genome of an adenovirus selected from among the canine, avian and human adenoviruses.

38. A complementation line according to claim 37, comprising notably a part of the genome of a type 5 human adenovirus.

39. A complementation line according to claim 38, comprising notably the part of the genome of a type 5 human adenovirus:

(i) extending from nucleotide 100 to nucleotide 5297;

(ii) extending from nucleotide 100 to nucleotide 4034; or

(iii) extending from nucleotide 505 to nucleotide 4034.

40. A complementation line according to claim 38 or 39, comprising notably the part of the E4 region of the genome of a type 5 human adenovirus extending from nucleotide 32800 to nucleotide 35826.

41. A complementation line according to claim 38, comprising notably the part of the genome of a type 5 human adenovirus extending from nucleotide 505 to nucleotide 35826.

42. A complementation line according to one of claims 32 to 41, comprising a part of the E1A region of the genome of an adenovirus deprived of its natural promoter; said part being placed under the control of an appropriate promoter.

43. A complementation line according to claim 42, in which said part of the E1A region is placed under the control of a promoter inducible by a nonadenoviral transcription trans-activating protein.

44. A complementation line according to claim 43, in which said part of the E1A region is placed under the control of a promoter inducible by a transcription trans-activating protein coded by an adenoviral vector according to claim 28 or 29.

45. A complementation line according to claim 43 or 44, in which said part of the E1A region is placed under the control of a promoter inducible by the *Saccharomyces cerevisiae* Gal4 transcription trans-activating protein.

46. A complementation line according to one of claims 32 to 45, comprising additionally a gene coding for a selection marker.

47. A complementation line according to claim 46, in which the selection gene codes for puromycin acetyl-transferase.

48. A complementation line according to claim 46 or 47, in which the selection gene is placed under the control of a promoter inducible by a transcription trans-activating protein coded by the E1A region of the genome of a wild-type adenovirus, notably under the control of the promoter of the E2 region of said genome.

49. A complementation line according to one of claims 32 to 48, derived from a pharmaceutically acceptable cell line.

50. A complementation line according to claim 49, derived from a cell line selected from among the Vero, BHK, A549, MRC5, W138 and CHO lines.

51. A complementation line according to one of claims 32 to 48, derived from a cell of the human embryonal retina.

52. A process for preparation of an adenovirus particle according to the invention, according to which:

(i) one introduces an adenoviral vector according to one of claims 1 to 29 into a complementation line capable of complementing *in trans* said adenoviral vector, so as to obtain a transfected complementation line;

(ii) one cultures said transfected complementation line under appropriate conditions to make possible the production of said adenovirus particle; and

(iii) one recovers said adenovirus particle in the cell culture.

53. A process according to claim 52, according to which one makes use of a complementation line according to one of claims 32 to 51.

54. Therapeutic or prophylactic use of an adenoviral vector according to one of claims 1 to 29, an adenovirus particle according to claim 30 or obtained by implementing a process according to claim 52 or 53, a eukaryotic host cell according to claim 31 or a complementation line according to one of claims 32 to 51.

55. A pharmaceutical composition comprising as therapeutic or prophylactic agent an adenoviral vector according to one of claims 1 to 29, an adenovirus particle according to claim 30 or obtained by implementing a process according to claim 52 or 53, a eukaryotic host cell according to claim 31 or a complementation line according to one of claims 32 to 51, in association with a pharmaceutically acceptable support medium.